An Analysis of Melanoprotein from Amphiuma Liver and from a Human Liver Melanoma.* (29244)

M. H. Hack and F. M. Helmy
Department of Medicine, Tulane University School of Medicine, New Orleans, La.

Our recent studies(1,2) have suggested that the melanoprotein normally found in the livers of amphibians and various other vertebrates may be an integral part of a hitherto unknown metabolic system peculiar to certain cold-blooded animals. Some of the analyses to be presented here indicate that the melanoprotein from 2 very divergent sources, amphiuma liver and human liver melanoma, are exceedingly similar. It is tempting to speculate that they have the same biochemical function in spite of the fact that the melanoma occurred in a tissue with high mitochondrial activity.

Materials and methods. Adult specimens of the amphibian *Amphiuma means* were killed by decapitation and the livers promptly removed and minced with scissors in ice-cold 0.25 M sucrose using 10 ml per gram of tissue. The ice-cold mince was then briefly homogenized in a Servall Omni-mixer followed by homogenization in an all-glass Potter-Elvehjem tissue grinder. Fat was removed from the homogenate by centrifuging at 25,000 $\times$ *g* for 10 minutes at 2°C. The supernatant was discarded and the precipitate washed 3 times in this way by resuspending in ice-cold 0.25 M sucrose after each centrifugation. Nuclei were then removed by centrifugation at 700 $\times$ *g* for 10 minutes. The supernatant was examined by phase contrast microscopy to determine the presence of nuclei; centrifugation at 700 $\times$ *g* was repeated if substantial numbers were observed. The melanoprotein granules in this supernatant were then sedimented by centrifugation at 1000 $\times$ *g* for 10 min and the resulting precipitate was washed twice with 0.25 M sucrose centrifuging at 1000 $\times$ *g* and twice with water (to remove the sucrose) centrifuging at 25,000 $\times$ *g*. The final black precipitate was freeze-dried.

The above isolation procedure was very wasteful since both the nuclear fraction and the 1000 $\times$ *g* supernatant were heavily contaminated by melanin granules, however, the melanoprotein fraction isolated was barely detectably contaminated by nuclei and mitochondria. The microsome fraction was effectively removed by the washing procedures.

The same procedure was employed for the isolation of melanoprotein granules from a sample of human liver melanoma obtained at autopsy (Tulane University No. A-63-1247). Because of the higher mitochondrial content of the starting sample, as detected by histochemical staining, the final melanoprotein fraction was probably not quite as homogeneous as the amphiuma liver fraction. The freeze-dried precipitate was dark-brown which was the color of the granules in unstained cryostat sections of the melanoma.

These freeze-dried melanoproteins were analyzed for their amino acid content and other ninhydrin reactive materials before and after bleaching by H_2O_2 in an attempt to separately assay the contribution of the protein and of the melanin as follows: a) 10-20 mg were hydrolyzed by 6 N HCl at 100°C for 24 hours in a tightly stoppered tube; the HCl was subsequently removed by a stream of N_2 with the tube immersed in a water bath at 100°. The amino acids were dissolved in 0.1 N HCl and the insoluble black humin removed by centrifugation. An aliquot of the supernate was then assayed for its ninhydrin reactive components using a Technicon Auto Analyzer.† b) 10-20 mg were bleached by reaction with 15% H_2O_2 at 60° for 6 hours, the insoluble protein component was separated by centrifugation at 700 $\times$ *g* for 15 minutes and the H_2O_2 soluble fraction was freeze-dried in order to remove the H_2O_2. Both fractions were then separately hydrolized by 6 N HCl as in a) above.

Because tryptophane is known to produce humin on reaction with hot HCl, one mg of

* Supported by U.S.P.H.S. grants.

† Instrument operated by R. A. Coulson and T. Hernandez, Louisiana State Univ. School of Med.

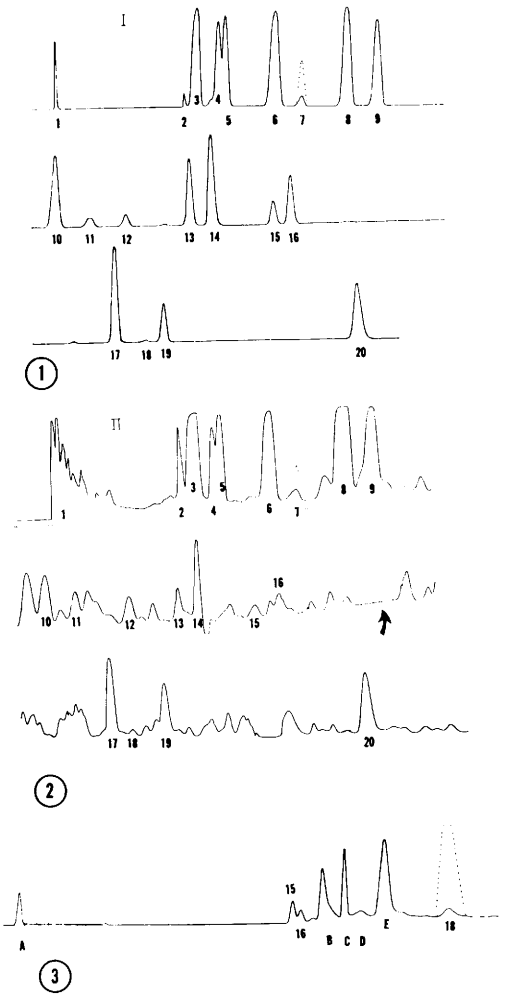

FIG. 1. 6 *N* HCl hydrolysate of amphiuma liver melanin pigment granules. The ninhydrin reactive materials were determined by a Technicon Auto Analyzer operating at a flow rate of 0.5 ml/min. Solid line is a tracing from the 570 mu colorimeter; dotted line from the 440 mu colorimeter. Numbered components are as follows: 1) cysteic acid, 2) hydroxyproline, 3) aspartic acid, 4) threonine, 5) serine, 6) glutamic acid, 7) proline, 8) glycine, 9) alanine, 10) valine, 11) cystine, 12) methionine, 13) isoleucine, 14) leucine, 15) tyrosine, 16) phenylalanine, 17) lysine, 18) tryptophane, 19) histidine, 20) arginine. Although ammonia was present on the original curve, between 16 and 17, it has been removed from this figure because it seemed to be primarily an artifact of analysis.

FIG. 2. 6 *N* HCl hydrolysate of H_2O_2 soluble portion of amphiuma liver melanin pigment granules. Same conditions of analysis as Fig. 1; the peak just preceding valine (#10) appears to be a hexose amine; arrow indicates the position ammonia occupied on original curve.

FIG. 3. Solid line shows ninhydrin positive products resulting from treatment of 1 mg of tryptophane in the same way as the melanin granules of tryptophane was processed as in b) above, and the amino acids identified with a Technicon amino acid analyzer with the hope that it would aid in interpretation of the melanin observations.

Claude(3) has reported data concerning the elementary composition of melanin granules isolated from amphiuma liver by differential centrifugation in saline. To permit comparison with his data, we submitted a sample of amphiuma liver melanoprotein to the Schwarzkopf Microanalytical Laboratory for analysis of P, S, Fe, and Cu. In addition, the lipid content was examined by paper chromatography and the PAS reaction was done on the H_2O_2 bleached granules because of our earlier observations(1) concerning the histochemical properties of the granules in cryostat sections. Cryostat sections of the melanoma were examined by the PAS reaction in order to further compare with the earlier(1) amphiuma work.

Results and discussion. Typical amino acid patterns of the amphiuma samples are shown in Fig. 1 and 2. Very similar curves were obtained from the melanoma where the same amino acids were represented. The large number of unknown components in the H_2O_2 samples of both amphiuma and melanoma cannot now be interpreted and there was no help from the tryptophane sample (Fig. 3). Cysteic acid was probably derived *in vitro* from cystine; there are very likely other *in vitro* products represented among the unknowns, many of which probably originated from the melanin. The insoluble nature of the humins formed did not permit a complete visualization of the hydrolytic products. All of the amino acids in the H_2O_2 bleached particles were also found in the H_2O_2 soluble fraction and probably represent degradation of the protein due to the action of the H_2O_2. Sometimes there was relatively more hexose

Fig. 2, *i.e.* oxidation by H_2O_2 followed by 6 *N* HCl hydrolysis. Dotted line is 1 mg of untreated tryptophane placed directly on the column in a separate analysis. Components appear to be: A) either aspartic acid or threonine, B) ethanolamine, C) ammonia, D) hydroxy lysine, E) 5-hydroxy tryptophane. About 60% of the nitrogen remained on top of column as insoluble humin. Flow rate of these 2 runs was 1 ml per min.

TABLE I. Analysis of Amphiuma Liver Melanoprotein Granules in % Dry Weight.

	Claude(3)	This paper
Phosphorus	.09	.59
Sulfur	2.16	1.30
Iron	.24	.10
Copper	.02	.08
Lipid	2.1	—

amine, tryptophane and phenylalanine in the H_2O_2 soluble fraction than in the bleached particles. The bleached granules from amphiuma liver and from the melanoma were both positive for the PAS (periodic acid Schiff) reaction in confirmation of the corresponding histochemical observations on cryostat sections(1).

The elementary analysis of the amphiuma liver sample is compared with Claude's(3) results in Table I; no comment concerning their differences is useful since the isolation techniques were different and because of expected normal variation in the chemical properties of the granules(1). The lipid paper chromatographic analysis of a chloroform-methanol extract of amphiuma liver melanoprotein showed sphingomyelin and monophosphoinositide and very little neutral lipid.

A preliminary series of histochemical observations were made on another human melanoma obtained as a surgical specimen and on a number of S-91, B-12, and H.P. mouse melanomas. The histochemical properties of the various granules in these melanomas, particularly as detected by the PAS reaction, resembled very closely those in amphiuma liver(1) and provide very encouraging evidence that the melanin pigment cell system described for amphiuma liver(1) is also operating in melanomas. Details will be reported later.

We have supplied Professor R. A. Nicolaus of the Institute of Organic Chemistry, University of Naples, with freeze-dried samples of amphiuma liver. According to Professor Nicholaus' analyses, amphiuma liver melanin is of the indole type; a report of his work will appear in Tetrahedron.

Mason *et al*(4) have reported 12 amino acids (not named) in acid hydrolysates of *Sepia* melanin assayed by paper chromatography. *Sepia* melanin has been extensively studied by Piattelli and associates(5).

Summary. Acid hydrolysates of melanoprotein isolated by differential centrifugation from amphiuma liver and from a human liver melanoma show them to be exceedingly similar with 20 amino acids identified. Bleaching of the melanoprotein by H_2O_2 fragmented both the melanin and the protein into a large number of unidentified ninhydrin reactive components. The bleached particle was positive to the PAS reaction.

We are indebted to T. D. McCaffery for technical assistance.

1. Helmy, F. M., Hack, M. H., *Acta Histochem.*, 1964, v17, 1.

2. Hack, M. H., Helmy, F. M., *ibid.*, 1964, in press.

3. Claude, A., *Harvey Lectures,* 1947-1948, v43, 121.

4. Mason, H. S., Ingram, D. J. E., Allen, B., *Arch. Biochem.*, 1960, v86, 225.

5. Piattelli, M. E., Fattorusso, E., Magno, S., Nicolaus, R. A., *Tetrahedron,* 1962, v18, 941.

Received February 10, 1964. P.S.E.B.M., 1964, v116.

An Assessment of the Role of Alpha and Beta Chylomicra in Hyperlipemic States.* (29245)

WILLIAM H. R. NYE (Introduced by Lawrence E. Young)
(Technical assistance by Gale Schultz)
Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

In alimentary lipemia and in the fasting hyperlipemia occurring in various disease states, chylomicra have been found to vary by electrophoresis, fatty acid analysis, and

* This work was supported by U.S.P.H.S. Research Grants.